Strigolactone promotes cytokinin degradation through transcriptional activation of *CYTOKININ OXIDASE/DEHYDROGENASE 9* in rice

Jingbo Duan[a,b,1], Hong Yu[a,b,1], Kun Yuan[a,b], Zhigang Liao[a,b], Xiangbing Meng[a,b], Yanhui Jing[a,b], Guifu Liu[a,b], Jinfang Chu[a,b], and Jiayang Li[a,b,c,2]

[a]State Key Laboratory of Plant Genomics, Institute of Genetics and Developmental Biology, The Innovative Academy of Seed Design, Chinese Academy of Sciences, 100101 Beijing, China; [b]National Center for Plant Gene Research (Beijing), Institute of Genetics and Developmental Biology, The Innovative Academy of Seed Design, Chinese Academy of Sciences, 100101 Beijing, China; and [c]College of Advanced Agricultural Sciences, University of Chinese Academy of Sciences, 100049 Beijing, China

Contributed by Jiayang Li, May 5, 2019 (sent for review June 27, 2018; reviewed by Motoyuki Ashikari and Harro J. Bouwmeester)

Strigolactones (SLs), a group of terpenoid lactones derived from carotenoids, are plant hormones that control numerous aspects of plant development. Although the framework of SL signaling that the repressor DWARF 53 (D53) could be SL-dependently degraded via the SL receptor D14 and F-box protein D3 has been established, the downstream response genes to SLs remain to be elucidated. Here we show that the cytokinin (CK) content is dramatically increased in shoot bases of the rice SL signaling mutant *d53*. By examining transcript levels of all the CK metabolism-related genes after treatment with SL analog GR24, we identified *CYTOKININ OXIDASE/DEHYDROGENASE 9* (*OsCKX9*) as a primary response gene significantly up-regulated within 1 h of treatment in the wild type but not in *d53*. We also found that OsCKX9 functions as a cytosolic and nuclear dual-localized CK catabolic enzyme, and that the overexpression of *OsCKX9* suppresses the browning of *d53* calli. Both the CRISPR/Cas9-generated *OsCKX9* mutants and *OsCKX9*-overexpressing transgenic plants showed significant increases in tiller number and decreases in plant height and panicle size, suggesting that the homeostasis of *OsCKX9* plays a critical role in regulating rice shoot architecture. Moreover, we identified the CK-inducible rice type-A response regulator *OsRR5* as the secondary SL-responsive gene, whose expression is significantly repressed after 4 h of GR24 treatment in the wild type but not in *osckx9*. These findings reveal a comprehensive plant hormone cross-talk in which SL can induce the expression of *OsCKX9* to down-regulate CK content, which in turn triggers the response of downstream genes.

strigolactone | cytokinin | hormonal crosstalk | *OsCKX9* | rice

Strigolatones (SLs), a group of carotenoid-derived plant hormones, are pivotal regulators of plant growth and development (1). Intensive research has revealed a conserved SL perception mechanism in plants. In rice (*Oryza sativa* L.), α/β hydrolase receptor DWARF 14 (D14) functions as both an SL receptor and an enzyme that binds and cleaves SL to trigger its conformational change (1). D14 interacts with F-box protein DWARF 3 (D3) and nuclear-localized repressor DWARF 53 (D53) in a SL-dependent manner, causing the ubiquitination and subsequent degradation of D53 within minutes (2, 3). D53 has been reported to act as a key component in the assembly of a repressor-corepressor-nucleosome complex via recruitment of the transcriptional corepressor TOPLESS (TPL) and TPL-related proteins (4). In the *d53* mutant, the dominant form of D53 is resistant to SL-induced degradation, and the SL signaling pathway is constitutively inhibited. Recently in rice, Ideal Plant Architecture 1 (IPA1) was identified as a D53-targeted downstream transcription factor that mediates SL-regulated tiller development and SL-induced *D53* expression (5). However, besides the feedback regulation of *D53*, the SL-responsive genes in rice are largely unknown but have long been speculated (1), especially the primary response genes and their downstream secondary response genes.

Cytokinins (CKs) constitute a class of plant hormones that play important roles in axillary bud initiation and outgrowth, as well as in various aspects of plant growth (6). CK and SL have shown antagonistic or combined/synergistic effects on several development processes. In pea (*Pisum sativum*), axillary bud outgrowth is induced by CK but inhibited by SL (7), and a similar phenomenon is seen in rice mesocotyl elongation (8). Repression of lateral root development by SL is abolished in CK-signaling mutants in *Arabidopsis thaliana* (9). It is speculated that SL and CK may share specific targets, or that one may regulate the key components in the metabolism or signaling pathway of the other. In pea, *BRANCHED 1* (*BRC1*) is activated by SL and repressed by CK (7, 10); however, in rice, *FINE CULM 1* (*FC1*), an orthologous gene of *BRC1*, is repressed by CK but insensitive to SL (11). The transcriptional regulation of SL and the cross-talk between SL and CK are not fully conserved between monocotyledonous and dicotyledonous plants, which remains to be elucidated.

In *Arabidopsis*, the type-A *Arabidopsis* response regulator (ARR) genes are rapidly induced by CK (12) and are considered negative-feedback regulators of CK signaling (13–17). Different type-A ARRs have been extensively studied in *Arabidopsis* for

Significance

Strigolactone plays a vital role in plant growth and development, but its response genes remain to be identified. In this study, we found that cytokinin content is markedly increased in the strigolactone signaling mutant *d53*, and that *OsCKX9*, which encodes a cytokinin oxidase to catalyze the degradation of cytokinin, functions as a primary strigolactone-responsive gene to regulate rice tillering, plant height, and panicle size, likely via a secondary response gene, *OsRR5*, which encodes a cytokinin-inducible rice type-A response regulator, demonstrating that strigolactone regulates rice shoot architecture through enhanced cytokinin catabolism by modulating *OsCKX9* expression.

Author contributions: J.D., H.Y., and J.L. designed research; J.D., K.Y., Z.L., X.M., Y.J., G.L., and J.C. performed research; J.D., H.Y., and J.L. analyzed data; and J.D., H.Y., and J.L. wrote the paper.

Reviewers: M.A., Nagoya University; and H.J.B., University of Amsterdam.

The authors declare no conflict of interest.

[1]J.D. and H.Y. contributed equally to this work.

[2]To whom correspondence may be addressed. Email: jyli@genetics.ac.cn.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1810980116/-/DCSupplemental.

Published online June 24, 2019.

their critical roles in diverse developmental processes (14, 15, 18–21). Comparatively, 13 type-A rice response regulators (OsRRs) have been annotated in the rice genome, but knowledge of their function is limited (22). Inhibition of CK signaling by *OsRR1* and *OsRR2* are known to be involved in rice crown root development (23, 24). Overexpression of *OsRR3*, *OsRR5*, and *OsRR6* can each inhibit the shoot regeneration from rice calli (25, 26). *OsRR6*-overexpressing transgenic plants exhibit dwarfism with small roots and inflorescences (26). These data suggest that *OsRR1*, *OsRR2*, *OsRR3*, *OsRR5*, and *OsRR6* are functionally negative regulators of CK signaling in rice.

In this study, we identify one primary SL-responsive gene, *OsCKX9*, which is rapidly induced by *rac*-GR24 in a D53-dependent manner and is likely responsible for the increased CK levels in *d53*. *OsCKX9* encodes a nuclear/cytosolic dual-localized CK catabolic enzyme. Both disruption and overexpression of *OsCKX9* could significantly increase tiller number, with reduced plant height and panicle size. Moreover, we show that *rac*-GR24 represses the expression of *OsRR5*, a CK-inducible rice type-A response regulator, and this repression is released in *OsCKX9* loss-of-function mutants, indicating that the CK signaling pathway is regulated by SL via OsCKX9. Collectively, these findings demonstrate that SLs directly active CK catabolism to regulate shoot architecture in rice.

Results

Shoot Bases of *d53* Have Elevated CK Levels. The local CK biosynthesis in tiller nodes is known to play an important role in bud elongation in rice (27, 28). To examine whether CK levels are altered in SL-related *dwarf* (*d*) mutants, we harvested the shoot bases of 20-d-old seedlings of the wild type (WT) and *d53*, and measured the endogenous concentrations of *trans*-zeatin (tZ) and N^6-(Δ^2-isopentenyl)adenine (2iP), two major forms of CK in rice (29), as well as their riboside derivatives tZR and iPR. The results showed significantly increased levels of tZ and 2iP in shoot bases of *d53*, ~2.0-fold and 1.3-fold higher, respectively, than those in WT (Table 1). For their riboside derivatives, iPR levels showed no significant difference between *d53* and WT, whereas the level of tZR in *d53* was ~1.4-fold higher than that in WT (Table 1). These results imply that the repression of the SL signaling pathway could result in an elevated CK content in rice shoot bases.

***OsCKX9* Acts as a Primary SL-Responsive Gene.** Previous research has shown that the CK levels are mainly determined by the balance between their biosynthesis and catabolism, which involves three biosynthetic enzymes—adenosine phosphate isopentenyltransferase (IPT), cytochrome P450 monooxygenase 735A (CYP735A), and the CK-activating enzyme LONELY GUY (LOG)—and one catabolic enzyme, cytokinin oxidase/dehydrogenase (CKX) (30). Since CK levels are increased in the shoot bases of *d53*, we asked whether SL could directly regulate the expression of CK biosynthesis or catabolism genes by quantifying the expression levels of all 34 CK metabolism genes. We found that *OsCKX9*, a CK catabolism gene, was significantly induced by *rac*-GR24 within 1 h (Fig. 1*A* and *SI Appendix*, Fig. S1), indicating that *OsCKX9* might be a primary SL-responsive gene.

D53 is a primary SL-responsive gene that is significantly down-regulated in rice SL-related *d* mutants, including SL biosynthesis mutants *d10*, *d17*, and *d27* and SL signaling mutants *d3*, *d14*, and *d53* (3). Similarly, we found that *OsCKX9* is significantly down-regulated in all these *d* mutants compared with WT (cv. Nipponbare) (Fig. 2*A*). We found that the OsCKX9 protein level was remarkably decreased in *d53* (Fig. 2*B*), in agreement with the decreased transcript level (Fig. 2*A*). We further examined the time course response of *OsCKX9* transcripts to *rac*-GR24 in shoot bases of *d27* and *d53* and found that *OsCKX9* is gradually up-regulated within 12 h in *d27* (Fig. 2*C*) but shows no response in *d53* (Fig. 2*D*). The foregoing results indicate that *OsCKX9* is a primary SL-responsive gene, and that the response of *OsCKX9* to SL requires the intact function of D53.

We further generated transgenic lines expressing *OsCKX9pro:GUS* and treated the transgenic lines at the seedling and heading stages with *rac*-GR24 for 12 h. Compared with the mock treatment, we observed enhancement in GUS activity in both *rac*-GR24–treated young seedlings and heading-stage plants (Fig. 2*E*), suggesting that *OsCKX9* expression is subject to SL regulation at different growth stages.

The rapid up-regulation of different CKXs by CK within 2 h was observed in *Arabidopsis*, maize, and rice, forming a negative feedback loop of the CK responses (31–33). However, this raises the question of whether *OsCKX9* expression is regulated by both SL and CK. Therefore, we treated rice seedlings with 6-benzylaminopurine (6-BA, a synthetic CK analog), tZ, or 2iP and quantified the expression levels of all 11 *OsCKX* genes in rice by qPCR. We found that 7 of the 8 detectable *OsCKX* genes (*OsCKX1*–*OsCKX5*, *OsCKX8*, and *OsCKX11*) were significantly induced by 6-BA, tZ, or 2iP, but the *OsCKX9* gene was not (Fig. 1 *C* and *D*). These results suggest that in CK catabolism, *OsCKX9* responds to SL rather than to CK.

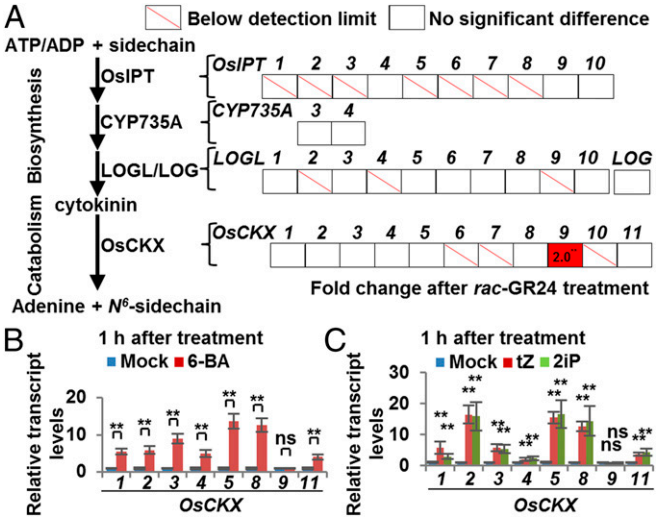

Fig. 1. Expression levels of CK metabolism genes on treatment with *rac*-GR24, 6-BA, tZ, or 2iP. (*A*) Fold change in CK metabolism gene expression levels after 1 h treatment with 5 μM *rac*-GR24 compared with mock. $^{**}P < 0.01$, Student's *t* test. (*B*) Expressions of eight *OsCKXs* on treatment with 5 μM 6-BA. Results are presented relative to mock. Values are mean ± SD, $n = 3$. $^{**}P < 0.01$ compared with mock, Student's *t* test; ns, no significant difference. (*C*) Expression levels of eight *OsCKXs* on treatment with 5 μM tZ or 2iP. Results are presented relative to mock. Values are mean ± SD, $n = 3$. $^{**}P < 0.01$ compared with mock, Student's *t* test; ns, no significant difference.

Table 1. CK content in the shoot bases of the WT and *d53*

	CK content, pg/mg fresh weight			
	tZ	tZR	2iP	iPR
WT	0.342 ± 0.038	0.651 ± 0.045	0.053 ± 0.006	0.258 ± 0.033
d53	0.681 ± 0.080**	0.902 ± 0.090**	0.067 ± 0.005**	0.202 ± 0.045^{ns}

Values are mean ± SD, $n = 6$. $^{**}P < 0.01$, Student's *t*-test; ns, no significant difference.

***OsCKX9* Encodes a Functional CKX Enzyme.** To examine the CK degradation activity of *OsCKX9*, the GST-OsCKX9 fusion protein was expressed in *Escherichia coli* cells and purified for in vitro assays (*SI Appendix*, Fig. S2). The CKX activity assays were carried out using tZ and 2iP as substrates, with 2,3-dimetoxy-5-methyl-1,4-benzoquinone

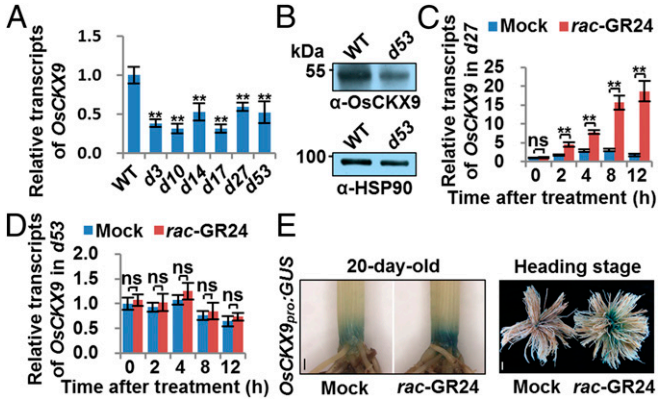

Fig. 2. *OsCKX9* is a primary strigolactone-responsive gene. (*A*) Expressions of *OsCKX9* in the WT and strigolactone-related *dwarf* mutants. Results are presented relative to WT. Values are mean ± SD, $n = 3$. $^{**}P < 0.01$, Student's *t* test. (*B*) OsCKX9 protein levels in WT and *d53*. α-OsCKX9 and α-HSP90, anti-OsCKX9 and anti-HSP90 antibodies. (*C* and *D*) Expression levels of *OsCKX9* on treatment with 5 μM *rac*-GR24 in *d27* (*C*) and *d53* (*D*). Results are presented relative to mock at 0 h. Values are mean ± SD, $n = 3$. $^{**}P < 0.01$, Student's *t* test; ns, no significant difference. (*E*) GUS staining with or without treatment with 5 μM *rac*-GR24 in $OsCKX9_{pro}$:*GUS* rice lines. (*Left*) Root-shoot junction of 20-d-old plants. (*Right*) Bottom view below the roots of heading-stage plants. (Scale bars: 1 mm in *Left*, 1 cm in *Right*.)

as the electron acceptor at pH 5.0 and 2,6-dichlorophenol indophenol as the electron acceptor at pH 7.5 using a modified endpoint method (34). The GST-OsCKX9 fusion protein exhibited relatively high activity for tZ at pH 5.0 (23.3 μkat g^{-1}), moderate activity for tZ at pH 7.5 (3.3 μkat g^{-1}), moderate activity for 2iP at pH 5.0 (3.7 μkat g^{-1}), and relatively low activity for 2iP at pH 7.5 (0.7 μkat g^{-1}) (Fig. 3*A*).

In *Arabidopsis*, overexpression of *CKXs* leads to CK-deficient phenotypes, including reduced shoot development, dwarfism, late-flowering, enhanced root growth, and reduced fertility (35). In rice, *ren1-D*, a dominant mutant of *OsCKX4*, exhibits decreases in plant height, tiller number, and primary and secondary branch number per panicle and an increase in crown root number (36), while the effects of other *OsCKX* genes remain elusive. Therefore, we generated two types of transgenic rice lines, one with Ubi_{pro}:*OsCKX9* expressing *OsCKX9* driven by the maize *ubiquitin 1* promoter and the other with Act_{pro}:*OsCKX9-GFP* expressing an *OsCKX9-GFP* fusion gene driven by the rice *actin 1* promoter. The *OsCKX9* transcript levels were up-regulated by 106-fold in Ubi_{pro}:*OsCKX9* and 56-fold in Act_{pro}:*OsCKX9-GFP* plants (Fig. 3*B*). Compared with WT (cv. Nipponbare), Act_{pro}:*OsCKX9-GFP* plants formed shorter culms, more tillers, and fewer primary and secondary branches per panicle and had a lower setting rate, similar to the morphological alterations caused by the CK deficiency in the *CKXs*-overexpressing plants in *Arabidopsis* (35). More severe defects were also observed in Ubi_{pro}:*OsCKX9* plants (Fig. 3*C* and *SI Appendix*, Table S1), possibly resulting from a higher expression level of *OsCKX9* in Ubi_{pro}:*OsCKX9*. Taken together, these results indicate that *OsCKX9* encodes a functional CKX enzyme in rice.

To identify the subcellular localization of the OsCKX9 protein, we analyzed the Act_{pro}:*OsCKX9-GFP* fluorescence pattern in roots and found that the OsCKX9-GFP proteins are expressed throughout the cytosol and accumulate mainly in nuclei (*SI Appendix*, Fig. S3). To confirm this, we transiently expressed a carboxyl-terminal eGFP fusion of OsCKX9 in rice protoplasts and found OsCKX9-eGFP protein accumulation predominantly in the cytosol and nuclei (Fig. 3*D*). These findings demonstrate that OsCKX9 is a cytosolic/nuclear dual-localized protein.

In plant tissue culture systems, a moderate concentration of CK is essential for shoot regeneration (37), and 6-BA treatment can result in the browning of calli (Fig. 3*E*). Overexpression of *OsCKX9* largely prevented the callus browning in WT treated with 6-BA (Fig. 3*E*), possibly because OsCKX9 degrades the endogenous CK in the callus and thus decreases the total amount of CK, including 6-BA. Inconsistent with the finding of elevated CK levels in *d53*, the calli derived from *d53* showed apparent browning (Fig. 3*E*). To test whether the browning of the *d53* calli is indeed caused by an increase in CK content, we generated transgenic *d53* calli expressing Ubi_{pro}:*OsCKX9* and found that the browning phenotype of *d53* calli could be largely rescued by overexpressing *OsCKX9* (Fig. 3*E*). Consistent with this, the expression levels of *OsRR1* and *OsRR2* were significantly increased in *d53* calli, which can be suppressed by overexpression of *OsCKX9* (*SI Appendix*, Fig. S4). These findings suggest that OsCKX9 is a functional CKX enzyme, and that its overexpression could rescue the browning of *d53* calli caused by an elevated CK level.

OsCKX9 Regulates Rice Plant Architecture. To further understand the biological function of OsCKX9, we investigated the spatial expression patterns of *OsCKX9* in various rice organs and found that *OsCKX9* is widely expressed in all the tissues at the heading stage, with the highest level in shoot bases (Fig. 4*A*). We then generated a loss-of-function mutant *osckx9* using CRISPR/Cas9 technology in the Nipponbare background. Sequence analysis revealed a 1-bp insertion at the first exon of *OsCKX9* in *osckx9*, which results in a premature stop codon (Fig. 4*B*). Indeed, absence of the OsCKX9 protein was confirmed by the immunoblotting analysis in *osckx9* (Fig. 4*C*). Compared with WT, *osckx9*

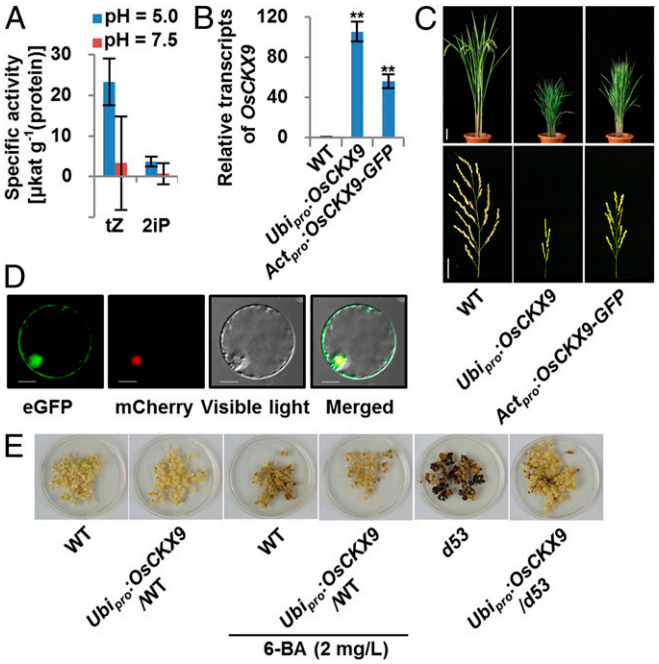

Fig. 3. *OsCKX9* encodes a functional CKX. (*A*) In vitro CKX activity assay of recombinant GST-OsCKX9. Values are mean ± SD ($n = 3$). (*B*) Expression levels of *OsCKX9* in WT, Ubi_{pro}:*OsCKX9*, and Act_{pro}:*OsCKX9-GFP* rice seedlings at the mature stage. Results are presented relative to WT. Values are mean ± SD, $n = 3$. $^{**}P < 0.01$, Student's *t* test. (*C*) Gross (*Upper*) and panicle (*Lower*) phenotypes of WT, Ubi_{pro}:*OsCKX9*, and Act_{pro}:*OsCKX9-GFP* at the mature stage. (*D*) Subcellular localization of OsCKX9-eGFP fusion protein in rice protoplasts. SV40NLS-mCherry is used to label the nucleus. (*E*) Phenotypes of calli derived from the WT, Ubi_{pro}:*OsCKX9*/WT, WT cultured with 6-BA, Ubi_{pro}:*OsCKX9*/WT cultured with 6-BA, *d53*, and Ubi_{pro}:*OsCKX9*/*d53*. (Scale bars: 10 cm in *C*, *Upper*; 3 cm in *C*, *Lower*; and 10 μm in *D*.)

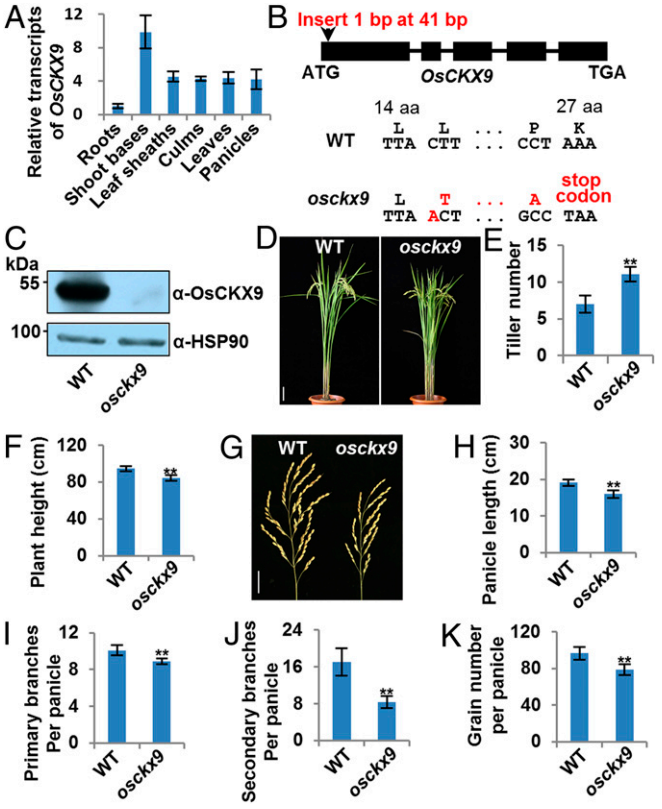

Fig. 4. Characterization of the *osckx9* mutant. (*A*) *OsCKX9* transcript levels in various organs at the heading stage. Results are presented relative to the expression level in roots. Values are mean ± SD; $n = 3$. (*B*) The mutation site in the *osckx9* coding region and its amino acid changes. (*C*) OsCKX9 protein levels in the WT and *osckx9*. α-OsCKX9 and α-HSP90, anti-OsCKX9 and anti-HSP90 antibodies. Molecular mass markers are shown on the left. (*D*) Phenotypes of WT and *osckx9* at the mature stage. (*E* and *F*) Comparisons of tiller number (*E*) and plant height (*F*) in *D*. (*G*) Panicles of WT and *osckx9* at the mature stage. (*H*–*K*) Comparisons of panicle length (*H*), primary branches per panicle (*I*), secondary branches per panicle (*J*), and grain number per panicle (*K*) in *G*. Values are mean ± SD, $n = 18$ (**$P < 0.01$; Student's *t* test). Bars = 10 cm in *D* and 5 cm in *G*.

displayed altered architecture in shoot and panicle (Fig. 4 *D* and *G*). The tiller number was significantly increased (Fig. 4*E*), while the plant height was slightly but significantly reduced in *osckx9* plants (Fig. 4*F*). For panicle morphology, *osckx9* showed reduced panicle length, primary and secondary branches per panicle, and grain number per panicle (Fig. 4 *H*–*K*). These results demonstrate that OsCKX9 regulates rice plant architecture at both the vegetative and reproductive stages.

OsCKX9 Works in the SL Signaling Pathway. In rice, SL deficiency leads to increased shoot branching and dwarfism. The *osckx9* plant exhibits similar phenotypes to *d27* but has less severe branching and dwarf stature than *d27* (Fig. 5 *A*–*C*). To further test whether OsCKX9 functions in the SL signaling pathway, we generated and characterized the *osckx9 d27* double mutant. As shown in Fig. 5 *A*–*E* and *SI Appendix*, Fig. S5, compared with *d27*, the *osckx9 d27* double mutant has similar tiller number, plant height, panicle length and branches, and grain number per panicle; however, compared with *osckx9* and WT, the double mutant is markedly increased in tiller number but decreased in other features, indicating that the *OsCKX9* functions in the SL signaling pathway as a downstream primary SL-responsive gene to regulate rice shoot architecture.

Based on the recent discoveries that IPA1 functions as a direct downstream transcription factor of D53 and that its transcriptional induction by SL depends on IPA1 in rice (5), we asked whether the *OsCKX9* induction by SL also requires IPA1 by examining the responses of *OsCKX9* transcripts to *rac*-GR24 in *ipa1-10*, a loss-of-function mutant of *IPA1* (5). As shown in Fig. 5*F*, on *rac*-GR24 treatment, the *OsCKX9* transcripts were significantly increased in *ipa1-10* plants, indicating that the SL-induced activation of *OsCKX9* is not mediated by IPA1. We also found that *rac*-GR24 could repress the tiller number of *osckx9* (Fig. 5*G*) but was unable to inhibit the bud outgrowth of *ipa1-10* (5), indicating that IPA1 is a crucial regulator in SL-induced rice tillering suppression. Taking together, these findings suggest that OsCKX9 works downstream in the SL signaling pathway but independently of IPA1.

Reduced CK Content by SLs Leads to Decreased *OsRR5* Expression. The expression levels of most type-A *OsRRs* are increased in leaves and roots after 6-BA treatment for 2 h, five of which—*OsRR1*, *OsRR2*, *OsRR3*, *OsRR5*, and *OsRR6*— have been reported to act as negative regulators in CK signaling to regulate various traits in rice (23–26). As SL could up-regulate the expression of *OsCKX9* in shoot bases (Figs. 1*B* and 2*C*), we wondered whether and which type-A *OsRRs* are in the downstream of CK catabolism regulated by SL. We first examined the expression levels of these five type-A *OsRRs* in the shoot bases of rice seedlings treated with 6-BA and found that *OsRR1*, *OsRR2*, *OsRR5*, and *OsRR6* were strongly induced by CK (*SI Appendix*, Fig. S6), but transcripts for *OsRR3* were undetectable. We then compared the expression levels of these four type-A *OsRRs* in the

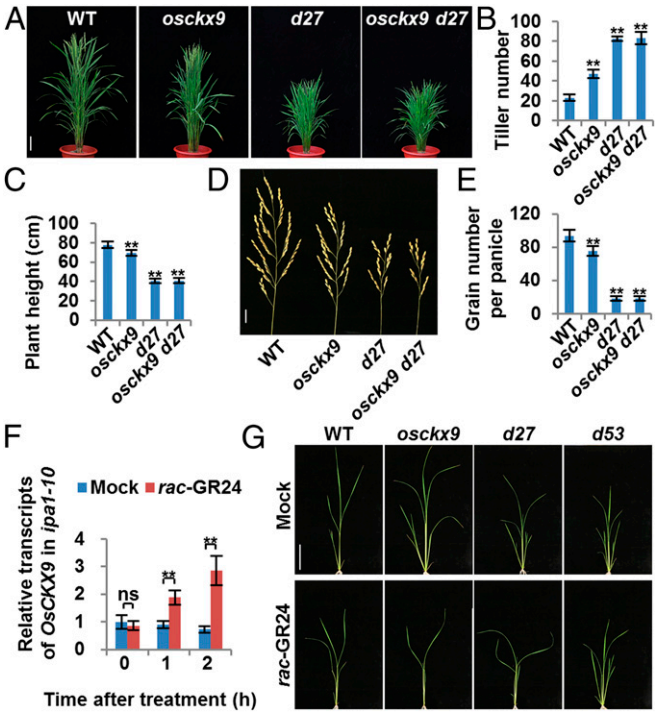

Fig. 5. Phenotypic comparison of *osckx9* and the strigolactone pathway mutants. (*A*) Phenotypes of the WT, *osckx9*, *d27*, and *osckx9 d27* at the heading stage. (*B* and *C*) Comparison of tiller number (*B*) and plant height (*C*) in *A*. (*D*) Panicles of WT, *osckx9*, *d27*, and *osckx9 d27* at the mature stage. (*E*) Comparison of grain number per panicle in *D*. Values are means ± SD, $n = 18$ (**$P < 0.01$; Student's *t* test). (*F*) Expressions of *OsCKX9* upon 5 μM *rac*-GR24 treatment in *ipa1-10*. Results are presented relative to mock at 0 h. Values are means ± SD, $n = 3$ (**$P < 0.01$; ns, no significant difference; Student's *t* test). (*G*) Responses of rice seedlings to 10 μM *rac*-GR24 treatment. Bars = 10 cm in *A* and *G* and 2 cm in *D*.

shoot bases of SL-related *d* mutants with the WT and found that *OsRR1*, *OsRR2*, and *OsRR6* showed no significant differences among these materials at transcript levels, but the expression of *OsRR5* was significantly higher in all the SL-related *d* mutants than in WT (Fig. 6*A*).

We tested whether the transcripts of *OsRRs* respond to SL using *D53* as a positive control (*SI Appendix*, Fig. S7*A*). Consistent with the results in SL-related *d* mutants, *OsRR5* expression was significantly repressed in shoot bases of WT after a 4-h treatment with *rac*-GR24 (Fig. 6*B*), while *OsRR1*, *OsRR2*, and *OsRR6* are all insensitive to *rac*-GR24 within 12 h (*SI Appendix*, Fig. S7 *B–D*). Furthermore, we found that the *rac*-GR24 repressed expression of *OsRR5* was released in shoot bases of the *osckx9* mutants (Fig. 6*C*), while this treatment resulted in pronounced induction of *D53* in shoot bases of the *osckx9* mutants (*SI Appendix*, Fig. S7*E*). These findings suggest that *OsRR5* is one of the downstream components of the CK catabolism regulated by SL-induced *OsCKX9*.

Discussion

Interactions between plant hormones are considered essential for their intact functions. SL and CK have been shown to function antagonistically in pea bud elongation (7, 10) and rice mesocotyl elongation in darkness (8); however, compared with the well-studied interactions between auxin and CK and between auxin and SL, relatively little is known about the interaction between CK and SL, especially in monocotyledonous plants. Here we report a comprehensive hormonal cross-talk between SL and CK in the shoot bases of rice. In response to *rac*-GR24, D53, the repressor of the SL signaling pathway, is degraded in minutes, leading to rapid transcriptional activation of the CK catabolism gene *OsCKX9* within 1 h and subsequent down-regulation of the CK-responsive gene *OsRR5* within 4 h, probably due to the OsCKX9-dependent degradation of CK (Fig. 6*D*). Consistent with these results, *OsCKX9* is largely down-regulated in all six SL-related *d* mutants tested in rice, resulting in an increased CK content. These findings demonstrate that SL could promote CK degradation through transcriptional activation of *OsCKX9* in rice. In *Arabidopsis*, *AtCKX1*- and *AtCKX3*-overexpressing plants exhibit multiple developmental alterations, including retarded shoot development and increased branch number, possibly resulting from decreased auxin content due to reduced auxin-producing tissues (35). However, in rice, overexpression and knockout of *OsCKX9* did not show opposite phenotype as expected, but both resulted in increased tiller number, reduced plant height, and decreased panicle size, suggesting that the phenotypes cannot be explained simply by the total CK content, instead pointing to a fine-tuned spatial and temporal-specific control of CK content in rice development.

Compared with other plant hormones that could trigger rapid responses of thousands of genes at the transcriptional level, surprisingly, significantly fewer genes are transcriptionally regulated after SL treatment over a short time frame (38, 39). One possibility is that significant responses to SL occur only in a very specific temporospatial manner. In rice, *D53* is the only reported gene that is rapidly up-regulated after SL treatment (2, 3). Here we demonstrate that *OsCKX9* is another primary SL-responsive gene downstream of *D53* in rice, the expression of which is significantly induced within 1 h after SL treatment (Fig. 1*B*). Consistent with the finding of elevated CK levels in *d53* (Table 1), it is likely that SL may constitutively regulate CK content by affecting *OsCKX9* homeostasis. Consistent with this, significantly elevated CK content at node 2 from the top of *D10*-RNAi plants has been reported (40). In addition, *OsCKX9* appears to be a special member of the *OsCKX* family, because its expression is insensitive to CK (Fig. 1 *C* and *D*) but rapidly induced by SL (Fig. 1*B*). It should be mentioned that the interactions of auxin with CK and auxin with SL have been established and proven to be critical for plant development, such as that the SL can inhibit auxin transport capacity and auxin inhibit CK biosynthesis (41). Therefore, along with the rapid induced activation of *OsCKX9*, SL may also affect CK content through hormonal cross-talk with auxin.

The transcriptional regulation of plant hormone action has been described as primary and secondary responses, with the former indicating a rapid protein synthesis-independent induction and the latter referring to an alteration depending on the products of the primary response genes (42, 43). *OsRR5* is significantly repressed by SL treatment after 4 h, and, more importantly, this repression depends on the intact function of the primary SL-responsive gene *OsCKX9* (Fig. 6*B*). Therefore, *OsRR5* functions as a secondary SL-responsive gene, which suggests that a secondary response program can occur as within 4 h, and that in such time, SL treatment could induce the transcriptional and translational responses of *OsCKX9*, the product of which could degrade CK and inhibit the *OsRR5* expression.

Different type-A *OsRRs* have overlapping/differential expression patterns in various organs (44), implying their diverse

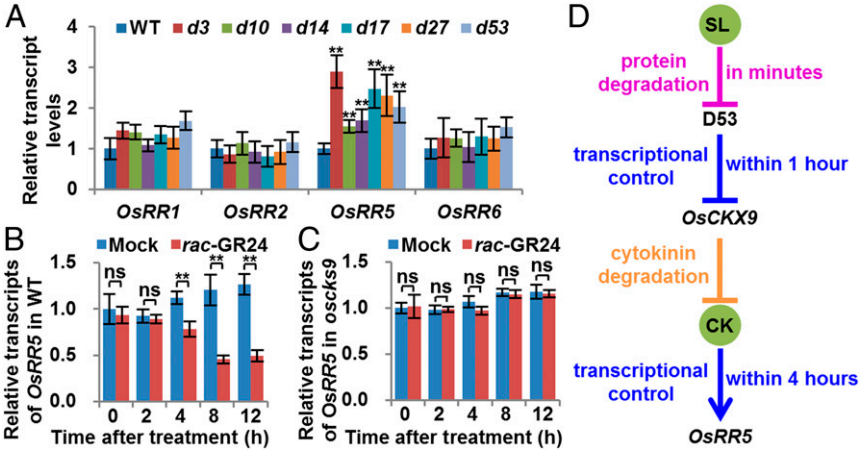

Fig. 6. *OsRR5* is a secondary strigolactone-responsive gene. (*A*) Expression levels of four *OsRRs* in the WT and strigolactone mutants. Results are presented relative to WT. Values are mean ± SD, $n = 3$. $^{**}P < 0.01$, Student's *t* test. (*B* and *C*) Expression levels of *OsRR5* on treatment with 5 μM *rac*-GR24 in WT (*B*) and *osckx9* (*C*). Results are presented relative to mock at 0 h. Values are mean ± SD, $n = 3$. $^{**}P < 0.01$, Student's *t* test; ns, no significant difference. (*D*) Proposed model of the OsCKX9-mediated strigolactone signaling pathway. In the shoot bases of rice, perception of strigolactone (SL) leads to degradation of D53, which in turn releases the repression of *OsCKX9* to degrade cytokinin (CK) and then triggers the reduction of *OsRR5*.

functions in specific organs. In shoot bases of rice, the expression of *OsRR5* was significantly up-regulated in all six SL-related *d* mutants (Fig. 6*A*) and inhibited by SL treatment (Fig. 6*B*), but the expression levels of *OsRR1*, *OsRR2*, and *OsRR6* were not (Fig. 6*A* and *SI Appendix*, Fig. S7 *B–D*), indicating that *OsRR5* specifically participates in SL-controlled CK responses in shoot bases. Corresponding to this, in green seedlings, the expression level of *OsRR5* is higher than that of the other type-A *OsRR* genes (44). CK is involved in various aspects of development, and *osckx9* plants display obvious alterations in plant height, tiller number, and panicle morphology (Fig. 4 *D–K*). It is quite possible that different type-A *OsRRs* may work in different organs downstream of SL-induced CK catabolism. A comprehensive dissection of the downstream genes will shed light on the regulation of different traits by SL-induced CK catabolism.

Identification of the transcription factors downstream of D53 is critical for understanding the SL signaling pathway. IPA1 has been reported as a direct downstream transcription factor regulated by D53, and the transcription activation activity of IPA1 can be suppressed by D53 (5), similar to AUXIN/INDOLE-3-ACETIC ACID INDUCIBLE proteins in the auxin-signaling pathway (45) and JASMONATE-ZIM DOMAIN proteins in the jasmonate-signaling pathway (46, 47). Although *rac*-GR24 can repress the tiller number of *osckx9*, it is unable to inhibit the bud outgrowth of *ipa1-10* (5), suggesting that IPA1 is a crucial regulator of SL-induced rice tillering suppression. A number of transcription factors have been revealed to be regulated by these hormonal repressors for their diverse functions (48, 49), but whether other transcription factors are regulated by D53 remains to be elucidated. Our finding that SL-induced activation of *OsCKX9* is dependent on D53 (Fig. 2*D*) but independent of IPA1 (Fig. 5*F*) demonstrates that other transcription factors besides IPA1 may function downstream of D53 in the SL signaling pathway, and these factors need to be identified for a better understanding of the SL signaling pathway.

Materials and Methods

Detailede information on plant growth, CK measurement, gene expression analysis, vector construction and plant transformation, GUS staining, antibody preparation, CKX activity assays, chemical treatment, and subcellular localization assays are provided in *SI Appendix, Materials and Methods*. The primers used in this study are listed in *SI Appendix*, Table S2.

ACKNOWLEDGMENTS. This work was supported by grants from the National Natural Science Foundation of China (31788103 and 91635301), the Chinese Ministry of Science and Technology (2016YFD0101800), and the Chinese Academy of Sciences (XDA08030101).

1. M. T. Waters, C. Gutjahr, T. Bennett, D. C. Nelson, Strigolactone signaling and evolution. *Annu. Rev. Plant Biol.* **68**, 291–322 (2017).
2. L. Jiang *et al.*, DWARF 53 acts as a repressor of strigolactone signalling in rice. *Nature* **504**, 401–405 (2013).
3. F. Zhou *et al.*, D14-SCF^{D3}-dependent degradation of D53 regulates strigolactone signalling. *Nature* **504**, 406–410 (2013).
4. H. Ma *et al.*, A D53 repression motif induces oligomerization of TOPLESS corepressors and promotes assembly of a corepressor-nucleosome complex. *Sci. Adv.* **3**, e1601217 (2017).
5. X. Song *et al.*, IPA1 functions as a downstream transcription factor repressed by D53 in strigolactone signaling in rice. *Cell Res.* **27**, 1128–1141 (2017).
6. E. Zürcher, B. Müller, "Cytokinin synthesis, signaling, and function—Advances and new insights" in *International Review of Cell and Molecular Biology*, K. W. Jeon, Ed. (Academic Press, 2016), **vol. 324**, pp. 1–38.
7. E. A. Dun, A. de Saint Germain, C. Rameau, C. A. Beveridge, Antagonistic action of strigolactone and cytokinin in bud outgrowth control. *Plant Physiol.* **158**, 487–498 (2012).
8. Z. Hu *et al.*, Strigolactone and cytokinin act antagonistically in regulating rice mesocotyl elongation in darkness. *Plant Cell Physiol.* **55**, 30–41 (2014).
9. L. Jiang *et al.*, Strigolactones spatially influence lateral root development through the cytokinin signaling network. *J. Exp. Bot.* **67**, 379–389 (2016).
10. N. Braun *et al.*, The pea TCP transcription factor PsBRC1 acts downstream of Strigolactones to control shoot branching. *Plant Physiol.* **158**, 225–238 (2012).
11. K. Minakuchi *et al.*, FINE CULM1 (FC1) works downstream of strigolactones to inhibit the outgrowth of axillary buds in rice. *Plant Cell Physiol.* **51**, 1127–1135 (2010).
12. I. Brandstatter, J. J. Kieber, Two genes with similarity to bacterial response regulators are rapidly and specifically induced by cytokinin in Arabidopsis. *Plant Cell* **10**, 1009–1019 (1998).
13. T. Kiba *et al.*, The type-A response regulator, ARR15, acts as a negative regulator in the cytokinin-mediated signal transduction in Arabidopsis thaliana. *Plant Cell Physiol.* **44**, 868–874 (2003).
14. J. P. C. To *et al.*, Type-A Arabidopsis response regulators are partially redundant negative regulators of cytokinin signaling. *Plant Cell* **16**, 658–671 (2004).
15. A. Leibfried *et al.*, WUSCHEL controls meristem function by direct regulation of cytokinin-inducible response regulators. *Nature* **438**, 1172–1175 (2005).
16. D. J. Lee *et al.*, Genome-wide expression profiling of ARABIDOPSIS RESPONSE REGULATOR 7 (ARR7) overexpression in cytokinin response. *Mol. Genet. Genomics* **277**, 115–137 (2007).
17. J. P. C. To *et al.*, Cytokinin regulates type-A Arabidopsis Response Regulator activity and protein stability via two-component phosphorelay. *Plant Cell* **19**, 3901–3914 (2007).
18. U. Sweere *et al.*, Interaction of the response regulator ARR4 with phytochrome B in modulating red light signaling. *Science* **294**, 1108–1111 (2001).
19. P. A. Salomé, J. P. C. To, J. J. Kieber, C. R. McClung, Arabidopsis response regulators ARR3 and ARR4 play cytokinin-independent roles in the control of circadian period. *Plant Cell* **18**, 55–69 (2006).
20. B. Müller, J. Sheen, Cytokinin and auxin interaction in root stem-cell specification during early embryogenesis. *Nature* **453**, 1094–1097 (2008).
21. W. Zhang, J. P. To, C. Y. Cheng, G. E. Schaller, J. J. Kieber, Type-A response regulators are required for proper root apical meristem function through post-transcriptional regulation of PIN auxin efflux carriers. *Plant J.* **68**, 1–10 (2011).
22. Y. Ito, N. Kurata, Identification and characterization of cytokinin-signalling gene families in rice. *Gene* **382**, 57–65 (2006).
23. Y. Kitomi *et al.*, The auxin responsive AP2/ERF transcription factor CROWN ROOTLESS5 is involved in crown root initiation in rice through the induction of OsRR1, a type-A response regulator of cytokinin signaling. *Plant J.* **67**, 472–484 (2011).
24. Y. Zhao *et al.*, The interaction between rice ERF3 and WOX11 promotes crown root development by regulating gene expression involved in cytokinin signaling. *Plant Cell* **27**, 2469–2483 (2015).
25. X. Cheng *et al.*, Overexpression of type-A rice response regulators, OsRR3 and OsRR5, results in lower sensitivity to cytokinins. *Genet. Mol. Res.* **9**, 348–359 (2010).
26. N. Hirose, N. Makita, M. Kojima, T. Kamada-Nobusada, H. Sakakibara, Overexpression of a type-A response regulator alters rice morphology and cytokinin metabolism. *Plant Cell Physiol.* **48**, 523–539 (2007).
27. Y. Liu *et al.*, Auxin inhibits the outgrowth of tiller buds in rice (Oryza sativa L.) by downregulating OsIPT expression and cytokinin biosynthesis in nodes. *Aust. J. Crop Sci.* **5**, 169–174 (2011).
28. J. Xu *et al.*, The interaction between nitrogen availability and auxin, cytokinin, and strigolactone in the control of shoot branching in rice (Oryza sativa L.). *Plant Cell Rep.* **34**, 1647–1662 (2015).
29. A. Osugi, H. Sakakibara, Q&A: How do plants respond to cytokinins and what is their importance? *BMC Biol.* **13**, 102 (2015).
30. T. Kudo, T. Kiba, H. Sakakibara, Metabolism and long-distance translocation of cytokinins. *J. Integr. Plant Biol.* **52**, 53–60 (2010).
31. A. Bhargava *et al.*, Identification of cytokinin-responsive genes using microarray meta-analysis and RNA-Seq in Arabidopsis. *Plant Physiol.* **162**, 272–294 (2013).
32. K. Podlešáková *et al.*, Novel cytokinin derivatives do not show negative effects on root growth and proliferation in submicromolar range. *PLoS One* **7**, e39293 (2012).
33. Y.-C. Tsai *et al.*, Characterization of genes involved in cytokinin signaling and metabolism from rice. *Plant Physiol.* **158**, 1666–1684 (2012).
34. I. Frébort *et al.*, Cytokinin oxidase/cytokinin dehydrogenase assay: Optimized procedures and applications. *Anal. Biochem.* **306**, 1–7 (2002).
35. T. Werner *et al.*, Cytokinin-deficient transgenic Arabidopsis plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. *Plant Cell* **15**, 2532–2550 (2003).
36. S. Gao *et al.*, CYTOKININ OXIDASE/DEHYDROGENASE4 integrates cytokinin and auxin signaling to control rice crown root formation. *Plant Physiol.* **165**, 1035–1046 (2014).
37. H. Motte, D. Vereecke, D. Geelen, S. Werbrouck, The molecular path to *in vitro* shoot regeneration. *Biotechnol. Adv.* **32**, 107–121 (2014).
38. S. M. Smith, J. Li, Signalling and responses to strigolactones and karrikins. *Curr. Opin. Plant Biol.* **21**, 23–29 (2014).
39. T. Waldie, H. McCulloch, O. Leyser, Strigolactones and the control of plant development: Lessons from shoot branching. *Plant J.* **79**, 607–622 (2014).
40. S. Zhang *et al.*, The interactions among DWARF10, auxin and cytokinin underlie lateral bud outgrowth in rice. *J. Integr. Plant Biol.* **52**, 626–638 (2010).
41. X. Cheng, C. Ruyter-Spira, H. Bouwmeester, The interaction between strigolactones and other plant hormones in the regulation of plant development. *Front. Plant Sci.* **4**, 199 (2013).
42. S. Abel, A. Theologis, Early genes and auxin action. *Plant Physiol.* **111**, 9–17 (1996).
43. B. Pauw, J. Memelink, Jasmonate-responsive gene expression. *J. Plant Growth Regul.* **23**, 200–210 (2004).
44. M. Jain, A. K. Tyagi, J. P. Khurana, Molecular characterization and differential expression of cytokinin-responsive type-A response regulators in rice (*Oryza sativa*). *BMC Plant Biol.* **6**, 1 (2006).
45. W. M. Gray, S. Kepinski, D. Rouse, O. Leyser, M. Estelle, Auxin regulates SCF$^{(TIR1)}$-dependent degradation of AUX/IAA proteins. *Nature* **414**, 271–276 (2001).
46. A. Chini *et al.*, The JAZ family of repressors is the missing link in jasmonate signalling. *Nature* **448**, 666–671 (2007).
47. B. Thines *et al.*, JAZ repressor proteins are targets of the SCF$^{(COI1)}$ complex during jasmonate signalling. *Nature* **448**, 661–665 (2007).
48. D. Weijers, D. Wagner, Transcriptional responses to the auxin hormone. *Annu. Rev. Plant Biol.* **67**, 539–574 (2016).
49. G. A. Howe, I. T. Major, A. J. Koo, Modularity in jasmonate signaling for multistress resilience. *Annu. Rev. Plant Biol.* **69**, 387–415 (2018).